Biodegradable Alginate Microspheres as a Delivery System for Naked DNA

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ABSTRACT

Sodium alginate is a naturally occurring polysaccharide that can easily be polymerized into a solid matrix to form microspheres. These biodegradable microspheres were used to encapsulate plasmid DNA containing the bacterial ß-galactosidase (LacZ) gene under the control of either the cytomegalovirus (CMV) immediate-early promoter or the Rous sarcoma virus (RSV) early promoter. Mice inoculated orally with microspheres containing plasmid DNA expressed LacZ in the intestine, spleen and liver. Inoculation of mice with microspheres containing both the plasmid DNA and bovine adenovirus type 3 (BAd3) resulted in a significant increase in LacZ expression compared to those inoculated with microspheres containing only the plasmid DNA. Our results suggest that adenoviruses are capable of augumenting transgene expression by plasmid DNA both in vitro and in vivo.

RÉSUMÉ

L'alginate de sodium est un polysaccharide naturel qui peut être facilement polymérisé en une matrice solide pour former des microsphères biodégradable. Des microsphères furent utilisées pour encapsuler de l'ADN plasmidique porteur du gène de la ß-galactosidase bactérienne (LacZ) sous contrôle du promoteur du cytomégalovirus (CMV) ou du virus du sarcome de Rous (VSR). Le gène LacZ fut exprimé dans l'intestin, la rate et le foie de souris inoculées par voie orale avec des microsphères contenant de l'ADN plasmidique. L'inoculation de souris avec des microsphères contenant l'ADN plasmidique et l'adénovirus bovin de type 3 causa une augmentation significative du niveau d'expression du LacZ comparativement au niveau exprimé chez des souris inoculées uniquement avec l'ADN plasmidique. Les résultats obtenus suggèrent que les adénovirus peuvent faire augmenter l'expression de transgène par l'ADN plasmidique autant in vitro que in vivo.

(Traduit par le docteur Serge Messier)

INTRODUCTION

Immunization with plasmid DNA encoding for the protein of interest has introduced a new concept in the area of vaccine development and gene therapy (1–4). In addition to injection of naked DNA into muscles (5,6), a number of delivery systems such as bombardment with gold microparticles coated with DNA (7–9), incorporation of DNA into liposomes and other polycationic lipids (10–12), biological erodable polymers (13), and others, have been evaluated for their transfection efficiency in cultured cells and animals. A number of heterologous regulatory sequences have been tested to determine their role in enhancing foreign gene expression in experimental animals inoculated with plasmid DNA. The cellular uptake of the naked DNA when injected into a muscle is significantly less, and following endocytosis a large portion of the DNA is degraded after fusion of endosomes (carrying the DNA) with lysosomes (14,15).

Following binding to specific cell-surface receptors (16,17), adenovirus is internalized by receptor-mediated endocytosis within a clathrin-coated vesicle. The endosomal membrane fuses with adenoviral capsid, triggered by exposure of hydrophobic residues of the adenoviral capsid proteins, due to a change in the endosomal pH (18). This process results in disruption of endosomes before they are fused with lysosomes. Conjugation of naked DNA with adenovirus using transferrin or polylysine results in internalization of these conjugates by receptor-mediated endocytosis and disruption of endosomes (19,20). It prevents lysis of DNA by lysosomal enzymes and thus provides higher levels of foreign gene expression.

Purified protein and viruses can be encapsulated in alginate microspheres (21,22). Oral immunization of mice, rabbits, and cattle with microspheres containing antigenic proteins elicited both humoral (mucosal and systemic) and cell-mediated immune responses. Since alginate microspheres are easy to prepare in large quantities and purified protein or live virus particles can be encapsulated, we wanted to determine whether this technology could be used for encapsulating plasmid DNA.

This paper describes the encapsulation of plasmid DNA into alginate microspheres as a vehicle for gene delivery. The use of bovine adenovirus type 3 (BAd3) to enhance the level of foreign gene expression

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in vivo by encapsulating the plasmid DNA mixed with BAd3 is also discussed.

MATERIALS AND METHODS

CELL CULTURE AND VIRUS

MDBK, PK-15, and 3T3 cells of bovine, porcine and murine origin, respectively, were obtained from American Type Culture Collection (ATCC), and grown as monolayer cultures using Eagle's minimum essential medium (MEM, Life Technologies Inc.) supplemented with 10% fetalClone III (HyClone Laboratories Inc.) and 50 μg/mL gentamicin. BAd3, obtained from ATCC, was grown in MDBK cells and purified by cesium chloride density-gradient centrifugation (23). The titer of the purified virus preparation was determined by plaque assay on MDBK cells.

PLASMIDS

Plasmids pTK21CMVgalSV40 (provided by Dr. Guo, Department of Veterinary Pathobiology, Purdue University, West Lafayette, Indiana) and pREP9gal (24) contain the bacterial ß-galactosidase (LacZ) gene under the control of the cytomegalovirus (CMV) immediate-early promoter and the Rous sarcoma virus (RSV) early promoter, respectively. The LacZ gene in both of these plasmid was under the control of the simian virus 40 (SV40) polyadenylation signal. Plasmid DNA was purified by isopycnic centrifugation in cesium chloride-ethidium bromide gradients (25).

TRANSFECTION OF CELLS

One day before transfection, semiconfluent monolayers of PK-15 and 3T3 cells were harvested with trypsin, counted, and 1 to 2 × 10^5 cells were added into each well of 6-well plates and incubated at 37°C in 5% CO_2. The cell monolayers were washed twice with OPTI-MEM I (Life Technologies Inc.). Plasmid DNA (1 or 5 μg) was mixed with 5 μg of Lipofectin (Life Technologies Inc.) and incubated at room temperature following the manufacturer's protocol. The DNA-liposome mixture was added dropwise to the cell monolayers, covered with OPTI-MEM I, and incubated for 30 min at 37°C. At this time point, a set of monolayers transfected with either pTK21CMVgalSV40 or pREP9gal were infected with BAd3 at a multiplicity of infection (m.o.i.) of 500 plaque forming units (pfu) per cell. At 24 h post-transfection, the medium was replaced with MEM containing 5% fetalClone III. The cells were harvested by scraping at 48 and 72 h post-transfection and the cell pellet was assayed for LacZ activity.

MICROENCAPSULATION OF PLASMID DNA AND BAD3

The protocol for preparing alginate microspheres was modified from a previously described procedure (21). To obtain a total of 10 mL of suspension of microspheres for each preparation, phosphate-buffered saline, (PBS) pH 7.2, 5 × 10^9 pfu purified preparation of BAd3, 1.5 mg pTK21CMVgalSV40, 1.5 mg pREPgal, 1.5 mg pTK21CMVgal SV40 + 5 × 10^9 pfu BAd3 or 1.5 mg pREPgal + 5 × 10^9 pfu BAd3 were mixed with sodium alginate solution and emulsified with oil to form microspheres which were stabilized by calcium chloride and zinc chloride. Thus, 1 mL suspension of microspheres may contain a maximum of either 5 × 10^8 pfu BAd3, 150 μg plasmid DNA, or 5 × 10^8 pfu BAd3 and 150 μg plasmid DNA. The majority of microspheres were within 5–10 μm in diameter as measured by Microtrak Particle Analyzer. All washings generated during the process of microencapsulation were collected and divided into 2 portions. One portion was extracted with phenol and chloroform, DNA was precipitated with ethanol and resuspended in a minimum volume of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Uncut DNA was run on an agarose gel by electrophoresis, stained with ethidium bromide and visualized under UV light. The second portion of all washings was centrifuged at 27 000 rpm for 2 h at 4°C in Beckman Ti50.1 rotor. The pellets were resuspended in minimum volumes of PBS and titrated for infectious virus particles by plaque assay on MDBK cells.

ANIMAL INOCULATION

A total of eighteen 6- to 8-week-old BALB/c mice were randomly grouped into 6 groups (3 animals per group) and were inoculated orally using a gavage needle and a syringe at Day 1, Day 2 and Day 3 with 1 mL suspension of alginate microspheres containing either PBS, BAd3, pTK21CMVgalSV40, pREP9gal, pTK21CMVgalSV40 + BAd3, or pREP9gal + BAd3. Animals were sacrificed at Day 5 by an overdose of sodium barbiturate and the small intestine, spleen, and liver were collected and divided into 2 portions. One portion of various tissues was weighed and used to assay for LacZ activity. The other portion was embedded in Tissue-Tek OTC compound (Miles Scientific, Inc.) and stored at −70°C until use. This portion was used for immunohistochemical and histochemical analyses.

ß-GALACTOSIDASE ASSAY

The protocol to measure LacZ activity was adapted from a previously described procedure (25). The cell pellets were resuspended in the cell extraction buffer (250 mM Tris-HCl (pH 7.8), 0.5% NP40, 1 mM phenylmethylsulfonylfluoride (PMSF)), vortexed, and the supernatants were saved for LacZ assay. The mouse tissues were homogenized in the cell extraction buffer using a tissumizer and the supernatants were used to assay for LacZ activity. A 40-μL sample of various dilutions of cell or tissue extracts was mixed with 350 μL of the sodium phosphate solution (100 mM sodium phosphate (pH 7.5), 10 mM KCl, 1 mM $MgSO_4$, and 50 mM 2-mercaptoethanol). After incubation at 37°C for 5 min, 132 μL of the ONGP solution (0.4% *o*-nitrophenol ß-D-galactopyranoside (ONGP) in 100 mM sodium phosphate, pH 7.5) was added and the incubation was continued for 1 h. The enzyme reaction was stopped with 172 μL of 1 M Na_2CO_3 and the intensity of the yellow color that developed was measured spectrophotometrically at 420 nm. Various dilutions of purified bacterial LacZ (Sigma Chemical Co., Inc.) were used as a standard for LacZ assay.

HISTOCHEMICAL STAINING

Frozen tissues sections were prepared using a cryomicrotome (Leica CM1800), were fixed with acetone and stored at −70°C. The histochemical assay to detect LacZ activity in situ was modified from a previously described protocol (26). Briefly, sections were cut from frozen tissues and fixed with 0.5% glutaraldehyde in

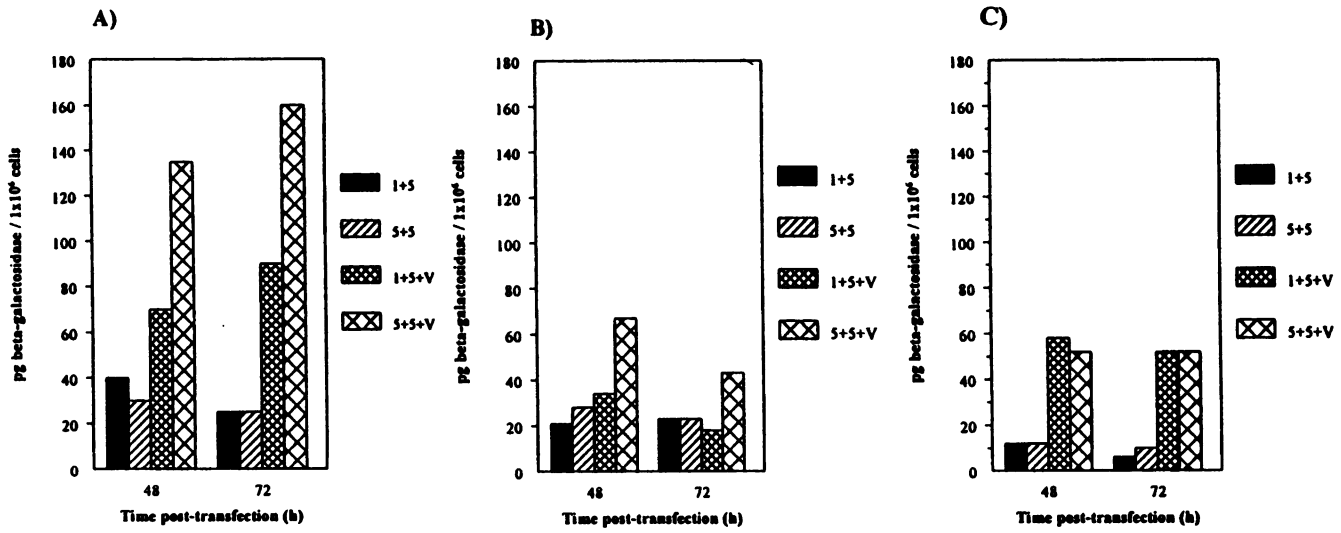

Figure 1. Expression of LacZ in cells initially transfected with plasmid DNA carrying the LacZ gene and subsequently infected with BAd3. PK-15, and 3T3 cells were transfected with either pTK21CMVgalSV40 or pREP9gal and subsequently infected with BAd3 at a m.o.i. of 500 pfu/cell. At 48 and 72 h post-transfection the cells were harvested, cell extracts were prepared and used to assay for LacZ activity. Mock and virus-infected cell extracts were used as negative controls. Purified bacterial LacZ was used as a standard. Each bar represents the mean of 2 independent samples. A) PK-15 cells transfected with pTK21CMVgalSV40, B) PK-15 cells transfected with pREP9gal, and C) 3T3 cells transfected with pTK21CMVgalSV40; 1 + 5, 1 μg DNA and 5 μg liposomes; 5 + 5, 5 μg DNA and 5 μg liposomes; 1 + 5 + V, 1 μg DNA + 5 μg liposomes + BAd3; 5 + 5 + V, 5 μg DNA + 5 μg liposomes + BAd3.

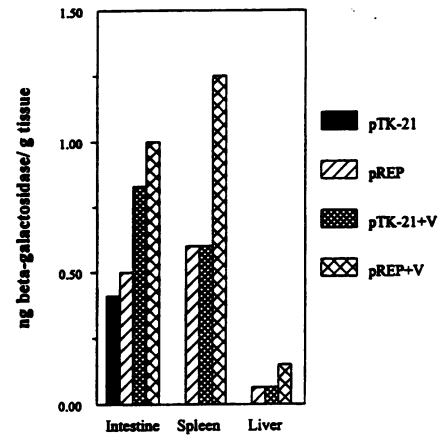

Figure 2. Expression of LacZ in tissues of mice inoculated with microspheres containing plasmid DNA and BAd3. The 6- to 8-week-old BALB/c mice were inoculated orally at Day 1, Day 2 and Day 3 with alginate microspheres containing either PBS, BAd3, pTK21CMVgalSV40, pREP9gal, pTK21CMVgalSV40 + BAd3, or pREP9gal + BAd3. At Day 5 post-inoculation, the small intestine, spleen and liver were collected and used to assay for LacZ activity. Purified bactrial LacZ was used as a standard. Each bar represents the mean value from 3 mice. pTK-21, pTK21CMVgalSV40; pREP, pREP9gal; pTK-21+V, pTK21CMVgalSV40 + BAd3; pREP+V, pREP9gal + BAd3.

PBS. After rinsing twice with PBS containing 1 mM $MgCl_2$, sections were overlayed with the X-gal solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal), 5.0 mM $K_3Fe(CN)_6$, 5.0 mM $K_4Fe(CN)_6$, and 1.0 mM $MgCl_2$ in PBS) and incubated overnight at room temperature. The slides were washed with PBS and counterstained with eosin. The blue deposits indicated LacZ activity.

RESULTS

ß-GALACTOSIDASE EXPRESSION IN CELLS TRANSFECTED WITH PLASMID DNA AND BAD3

We tested the effciency of 2 heterologous promoters, the cytomegalovirus (CMV) immediate-early promoter and the Rous sarcoma virus (RSV) early promoter, to drive transient expression of the LacZ gene when cells initially transfected with plasmid DNA are subsequently infected with BAd3. Since BAd3 does not replicate in either PK-15 cells or 3T3 cells (data not shown), virus infection of these cells will not cause cell lysis. Expression of LacZ in PK-15 cells transfected with pTK21CMVgalSV40 or pREPgal and subsequently infected with BAd3 was approximately 1.7 to 6.4- or 0.7 to 2.3-fold higher, respectively, compared to transfected cells without virus infection (Fig. 1A; 1B). Similarly, expression of LacZ in 3T3 cells transfected with pTK21CMVgalSV40 and subsequently infected with BAd3 was approximately 4.3 to 8.6-fold higher compared to transfected cells without virus infection (Fig. 1C). We did not get detectable levels of LacZ expression with pREPgal in 3T3 cells under the conditions used for pTK21CMVgalSV40. The maximum level of LacZ expression with pREPgal + BAd3 even in PK-15 cells was approximately half compared to that of pTK21CMVgalSV40 + BAd3. Expression of LacZ in 3T3 cells with pTK21CMVgalSV40 + BAd3 was approximately half of that obtained in PK-15 cells. The choice of both promoter and cell line seems to be responsible for the levels of reporter gene expression in a transient expression assay. Expression of LacZ with a plasmid carrying the LacZ gene under the control of the mouse cytomegalovirus (MCMV) immediate-early promoter was approximately 3 times higher than that with the human CMV promoter (unpublished results). We did not find reporter gene expression proportionate to the amount of plasmid DNA used. This was mainly due to limiting amounts of liposomes when higher amounts of plasmid DNA were used, since by increasing quantities of liposomes with increasing amounts of DNA, the levels of foreign gene expression were proportionate to the amount of DNA used (data not shown). However, this does not have a significant effect on the interpretation of our results.

MICROENCAPSULATION OF PLASMID DNA AND BAD3

In order to determine whether alginate microspheres could be used for encapsulating plasmid DNA for gene delivery and effect of BAd3 on level of foreign gene expression by plasmid DNA in vivo, we generated microspheres containing either plasmid DNA, virus or both. None of the washings showed DNA in detectable amounts (data not shown) indicating that the efficiency of microencapsulation of plasmid DNA was high. Infectious virus titers from various washings were insignificant (data not shown). Since the process of microencapsulation did not involve any treatment that obviously could decrease virus infectivity, we assumed that the microencapsulation of BAd3 was also efficient.

EXPRESSION OF ß-GALACTOSIDASE IN TISSUES OF MICE INOCULATED WITH MICROSPHERES

Mice were orally inoculated with microspheres containing either plasmid DNA, virus or both and the intestine, spleen and liver were collected and analyzed for LacZ expression. In mice inoculated with microspheres containing only pTK21CMVgalSV40, reporter gene expression was observed in intestine, whereas in mice receiving microspheres containing pTK21CMVgalSV40 and BAd3, LacZ expression was observed in intestine, spleen and liver (Fig. 2). In mice inoculated with microspheres containing either pREPgal or pREPgal + BAd3, LacZ expression was observed in intestine, spleen and liver (Fig. 2). Expression of reporter gene was comparatively less in liver with either plasmid. In mice inoculated with plasmid DNA and virus, expression of LacZ in intestine, spleen and liver was approximately 2-fold higher than the expression obtained with the plasmid DNA only.

The intestine, spleen and liver sections from mice inoculated with microspheres containing plasmid DNA and BAd3 were analyzed for LacZ expression by cytochemical staining. The reporter gene expression was evident from blue color development in some cells in all 3 tissues tested (Fig. 3 D, E, F). None of the control tissues had color development above background (Fig. 3 A, B, C).

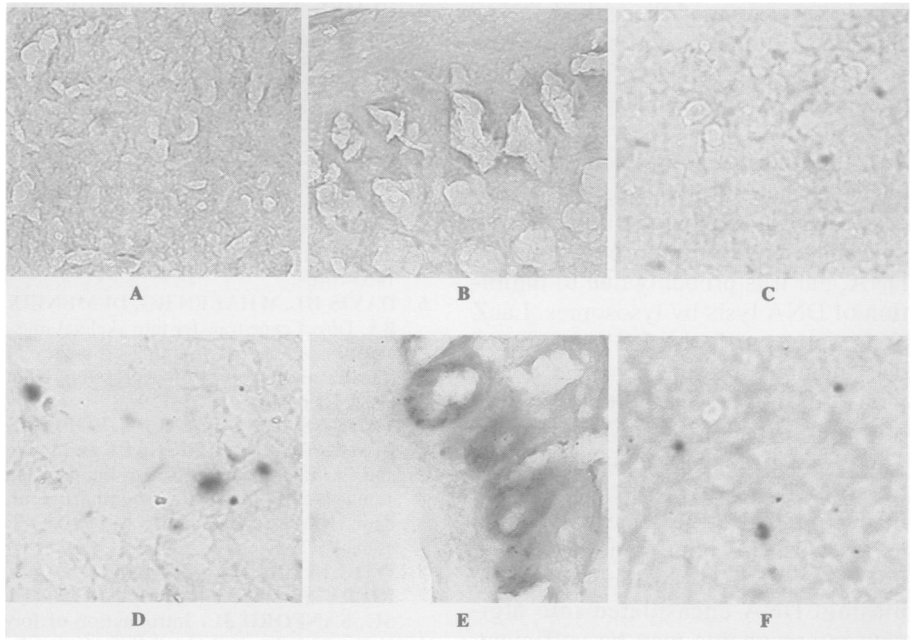

Figure 3. Histochemical analysis for LacZ in tissues of mice inoculated with microspheres containing plasmid DNA and BAd3. The 6- to 8-week-old BALB/c mice were inoculated orally at Day 1, Day 2 and Day 3 with alginate microspheres containing either BAd3 or pTK21CMVgalSV40 + BAd3. At Day 5 post-inoculation, the small intestine, spleen and liver were collected and embedded in OTC compound. Frozen sections were analyzed for LacZ expression using X-gal. A) liver, B) intestine, and C) spleen sections from the animal inoculated with BAd3. D) liver, E) intestine, and F) spleen sections from the animal inoculated with pTK21CMVgalSV40 + BAd3.

Similarly, the intestine, spleen and liver sections from mice inoculated with microspheres containing plasmid DNA and virus were analyzed for expression of LacZ by immunohistochemical staining using a monoclonal antibody against LacZ. The reporter gene expression was evident from brown color deposits in some cells in all 3 tissues tested (data not shown). As expected, control tissues did not develop color above background. Since cytochemical and immunohistochemical examinations were not quantitative, mouse tissues were not analyzed from all groups.

DISCUSSION

The efficiency of naked DNA entry into cells and its survival in the cytoplasm before it reaches the nucleus are 2 important factors that directly affect levels of transgene expression in addition to the choice of heterologous regulatory sequences. Receptor-mediated endocytosis could enhance the efficiency of plasmid DNA entry into cells (19,20). Various mechanisms that protect DNA from degradation within the cytoplasm will ensure transport of the maximum amount of DNA into the nucleus for transcription. In order to extend the range of delivery systems for the naked DNA, we encapsulated the plasmid DNA into alginate microspheres. Our results have 2 important findings: i) oral inoculation of mice with alginate microspheres containing plasmid DNA led to transgene expression in the intestine, spleen and liver, and ii) microspheres containing both the naked DNA and BAd3 caused enhanced reporter gene expression in vivo compared to that of microspheres containing only plasmid DNA.

Since adenovirus can efficiently enter a number of both replicating and quiescent cells, it may be used as a mediator for macromolecular transport into cells. Exposure of cultured cells to human adenovirus and unmodified plasmid DNA led to cointernalization of the plasmid DNA and the virus by receptor-mediated endocytosis (27). This resulted in augmentation of the transgene gene expression compared to the cells exposed with the plasmid DNA alone. Since BAd3 does not replicate in cultured cells of murine or porcine origin, it was necessary to determine whether BAd3 entry into these cells will take place, resulting in enhancement in

gene expression of the naked DNA. There was an increase in LacZ expression with BAd3 in cells transfected with either pTK21CMVgalSV40 or pREPgal.

We did not attempt to determine the exact mechanism involved in the process of BAd3-mediated augmentation of transgene expression by the naked DNA, but it is probably due to inhibition of DNA lysis by lysosomes. LacZ expression with the RSV promoter was undetectable in 3T3 cells but in mouse tissues it was better than that obtained with the CMV promoter. Therefore, it appears that there could be significant differences between in vitro and in vivo gene expression levels with the same gene construct. For many applications the use of only plasmid DNA encasulated into alginate microspheres may be sufficient, however, plasmid DNA could be mixed with an adenovirus if a higher level of transgene gene expression is required. Currently experiments are in progress to determine whether encapsulation of adenovirus with plasmid DNA affect levels and type of immune response elicited against the transgene product. This novel gene delivery system has potential for immunization and gene therapy applications.

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REFERENCES

1. **ULMER JB, DONNELLY JJ, PARKER SE, et al.** Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259: 1745–1749.
2. **ULMER JB.** Elegantly presented DNA vaccines. Nat Biotechnol 1997; 15: 842–843.
3. **DONNELLY JJ, ULMER JB, LIU A.** DNA vaccines. Life Sci 1997; 60: 163–172.
4. **FELGNER PL.** Nonviral strategies for gene therapy. Sci Am 1997; 276: 102–106.
5. **WOLFF JA, MALONE RW, WILLIAMS P, et al.** Direct gene transfer into mouse muscle in vivo. Science 1990; 247: 1465–1468.
6. **DAVIS HL, WHALEN RG, DEMENEIX BA.** Direct gene transfer into skeletal muscle in vivo: factors affecting efficiency of transfer and stability of expression. Hum Gene Ther 1993; 4: 151–159.
7. **YANG NS, BURKHOLDER J, ROBERTS B, MARTINELL B, MCCABE D.** In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc Natl Acad Sci USA 1990; 87: 9568–9572.
8. **WILLIAMS RS, JOHNSTON SA, RIEDY M, DEVIT MJ, MCELLIGOTT SG, SANFORD JC.** Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc Natl Acad Sci USA 1991; 88: 2726–2730.
9. **TANG D, DEVIT M, JOHNSTON SA.** Genetic immunization is a simple method for eliciting an immune response. Nature 1992; 356: 152–154.
10. **FELGNER PL, RINGOLD GM.** Cationic liposome-mediated transfection. Nature 1989; 337: 387–388.
11. **BARTHEL F, REMY JS, LOEFFLER JP, BEHR JP.** Gene transfer optimization with lipospermine-coated DNA. DNA Cell Biol 1993; 12: 553–560.
12. **DEMENEIX BA, ABDEL-TAWEB H, BENOIST C, SEUGNET I, BEHR JP.** Temporal and spatial expression of lipospermine-compacted genes transferred into chick embryos in vivo. Biotechniques 1994; 16: 496–501.
13. **MATHIOWITZ E, JACOB JS, JONG YS, et al.** Biologically erodable microspheres as potential oral drug delivery systems. Nature 1997; 386: 410–414.
14. **ZENKE M, STEINLEIN P, WAGNER E, COTTEN M, BEUG H, BIRNSTIEL ML.** Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. Proc Natl Acad Sci USA 1990; 87: 3655–3659.
15. **COTTEN M, WAGNER E, ZATLOUKAL K, PHILLIPS S, CURIEL DT, BIRNSTIEL ML.** High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome disruption activity of defective or chemically inactivated adenovirus particles. Proc Natl Acad Sci USA 1992; 89: 6094–6098.
16. **BERGELSON JM, CUNNINGHAM JA, DROGUETT G, et al.** Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 1997; 275: 1320–1323.
17. **HONG SS, KARAYAN L, TOURNIER J, CURIEL DT, BOULANGER PA.** Adenovirus type 5 fiber knob binds to MHC class I alpha2 domain at the surface of human epithelial and B lymphoblastoid cells. EMBO J 1997; 16: 2294–2306.
18. **SETH P.** Mechanism of adenovirus-mediated endosome lysis: role of the intact adenovirus capsid structure. Biochem Biophy Res Comm 1994; 205: 1318–1324.
19. **CURIEL DT, AGARWAL S, WAGNER E, COTTEN M.** Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. Proc Natl Acad Sci USA 1991; 88: 8850–8854.
20. **COTTEN M, WAGNER E, BIRNSTIEL ML.** Receptor-mediated transport of DNA into eukaryotic cells. Methods Enzymol 1993; 217: 618–644.
21. **BOWERSOCK TL, HOGENESCH H, SUCKOW M, et al.** Oral vaccination with alginate microsphere systems. J Cont Rel 1996; 39: 209–220.
22. **BOWERSOCK TL, HOGENESCH H.** Oral immunization using microparticles. In: Park K, ed. Controlled Drug Delivery Challenges and Strategies. Washington: American Chemical Society, 1997: 269–288.
23. **GRAHAM FL, PREVEC L.** Manipulation of adenovirus vectors. In: Murray EJ, ed. Methods in Molecular Biology: Gene Transfer and Expression Protocols, Vol. 7. Clifton: The Humana Press, 1991: 109–128.
24. **SCHOLZ E, ZHANG C, GUO P.** Transactivation of the early SV40 promoter by avian infectious laryngotracheitis virus in avian cells. J Virol Methods 1993; 45: 291–301.
25. **SAMBROOK J, FRITSCH EF, MANIATIS T.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.
26. **ENGELHARDT JF, YE X, DORANZ B, WILSON JM.** Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc Natl Acad Sci USA 1994; 91: 6196–6200.
27. **YOSHIMURA K, ROSENFELD MA, SETH P, CRYSTAL RG.** Adenovirus-mediated augmentation of cell transfection with unmodified plasmid vectors. J Biol Chem 1993; 268: 2300–2303.